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STEROID–PHOSPHATIDYLCHOLINE INTERACTIONS IN ORIENTED MULTIBILAYERS—A SPIN LABEL STUDY

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SUMMARY

1. The effects of incorporated cholesterol, and structural derivatives, upon the molecular motion of egg phosphatidylcholine in oriented multibilayers has been investigated using spin labelled lipid probes.

2. Addition of cholesterol to the multibilayers decreases the amplitude of motion of the lipid probes, indicating an increase in order of the phospholipids.

3. Cholesterol, lathosterol and 7-dehydrocholesterol are as effective as cholesterol in reducing the random motion of the bilayer phospholipids.

4. Cholesteryl methyl ether, cholesteryl chloride, 4-cholesten-3-one, thiocholesterol and epicholesterol have no significant ordering effect.

5. The plant sterols ergosterol and β-sitosterol have little ordering effect while sterols lacking the C-17 side chain, 5α-androstane-3β-ol and 5α-androstane-3β-ol-17-one, produce no detectable effect.

6. For maximum interaction with phosphatidylcholine a steroid with a planar nucleus requires a C-3 β-hydroxyl group and an iso-octyl group at C-17, indicating the importance of stereospecific polar and hydrophobic forces in steroid–phospholipid interactions.

INTRODUCTION

Cholesterol is a major constituent of many cell membranes but the nature of its association with the phospholipid components is not well understood. If mechanisms proposed for the biological activity of cholesterol and related steroids are to have a plausible structural basis, detailed knowledge of the molecular forces involved in phospholipid–sterol interactions is essential.

Spin labelled lipid probes are extremely useful for deriving information about molecular orientation and motion of phospholipids in biological and model membrane systems[1–5]. It has been shown that the motion of spin labels in model membrane systems is sensitive to the presence of cholesterol[6–14]. Using planar phospholipid multibilayers, it has been demonstrated that the changes in the resonance spectra of

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intercalated spin labels upon addition of cholesterol could be correlated with a reduction in the random thermal motion of the phospholipids[10,11,14], (*i.e.* an increase in order). The decrease in the thermal motion of the bilayer components results in a decrease in the surface area occupied by each lipid[15,16] (a condensing effect) which in turn causes an increase in the bilayer thickness[17,18], altering its permeability properties[19,20]. A comparison of the effects of cholesterol and structural derivatives on the motion of spin labelled lipids in the oriented multibilayers provides a convenient method for obtaining information concerning sterol–phospholipid interactions. Previous results indicated the existence of stereospecific hydrophobic and polar interactions between cholesterol and phospholipids[11,12].

In this paper we present a comparative study of the ordering effects of cholesterol and twelve related steroids in oriented egg phosphatidylcholine multibilayers. The effects of these steroids were monitored with two spin labels; 3-spiro-[2′-(*N*-oxyl-4′,4′-dimethyloxazolidine)] cholestane, (3-doxylcholestane), and 12-spiro-[2′-(*N*-oxyl-4′,4′-dimethyloxazolidine)] stearic acid, (12-doxylstearic acid). The results indicate that solubility of steroids and degree of order induced by them in the phospholipid films is dependent on the polarity and configuration of the C-3 and C-17 substituents. The degree of saturation of the B-ring has only minor influence on the ordering capability of a sterol.

MATERIALS AND METHODS

Lipids

The steroids were purchased from Mann Research Laboratories and Sigma Chemical Co. Steroids which did not have sharp melting points were recrystallized from methanol until the maximum melting range was ± 1 °C. Egg phosphatidylcholine was purified by column and thin layer chromatography. 3-Spiro-[2′-(*N*-oxyl-4′,4′-dimethyloxazolidine)] cholestane was prepared by the method of Keana *et al.*[21]. 12-Spiro-[2′-(*N*-oxyl-4′,4′-dimethyloxazolidine)] stearic acid was synthesized by the procedure of Waggoner *et al.*[8].

Oriented multibilayers

Thin lipid films containing a fixed egg lecithin to spin label ratio of 150:1 were prepared as reported previously[10]. The multibilayer films were formed by evaporating a chloroform solution of the lipids under reduced pressure in a standard ESR quartz aqueous cell. Each film preparation contained 1–2 mg of phosphatidylcholine. The lipid films were hydrated by adding 500 μl of 0.15 M NaCl aqueous solution to the cell.

ESR measurements

The resonance spectra of the spin labels in hydrated multilayers were recorded with the plane of the flat quartz cell parallel and perpendicular to the magnetic field vector. All spectra were recorded at room temperature on a Varian E3 or E6 spectrometer. The data reported are the average of at least two experiments. The maximum deviation in the experimental hyperfine splittings of a spin label in different films of the same composition was ± 0.2 G.

Spectral analysis

The analysis of the resonance spectra of spin labels in bilayer systems has been discussed by several authors[22-27]. In this paper we have used the semi-quantitative treatment of the motion of spin labels introduced by McConnell[28]. A very thorough discussion of this theory as applied to oriented multilayers is given by Seelig[24]. The degree of order reported by a spin label may be defined by an order parameter S_3[29,30].

$$S_3 = \frac{T'_{\parallel} - T'_{\perp}}{T_{zz} - T_{xx}} \tag{1}$$

S_3 is a measure of the degree of alignment of the z axis of the nitroxide with the normal of the bilayer plane. $T'_{\parallel}$ and $T'_{\perp}$ are the experimentally observed hyperfine splittings when the normal of the bilayer is parallel or perpendicular to the applied magnetic field direction. The nitroxide tensor components T_{zz} and T_{xx} are calculated as described by Seelig[24].

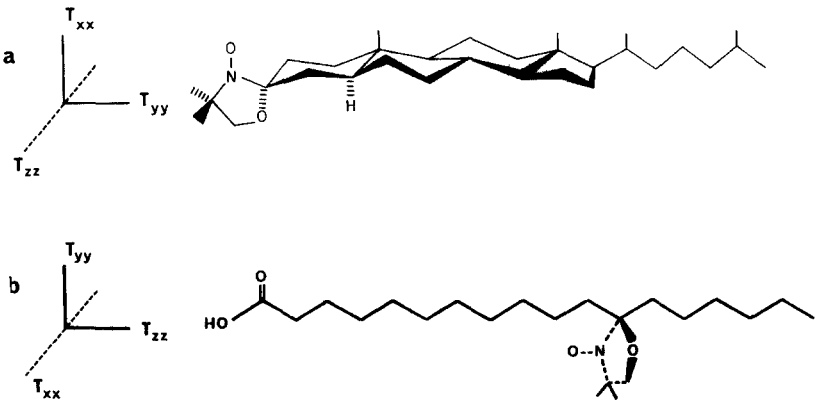

Fig 1 (a) 3-doxylcholestane T_{yy} is essentially parallel to the molecular long axis (b) 12-doxylstearic acid in the all-*trans* configuration T_{zz} is parallel to the molecular long axis

The y axis of the nitroxide moiety in the cholestane spin label is essentially parallel to the rigid steroid long axis (Fig. 1). When there is rapid axial rotation of the label ($\gg 73 \cdot 10^6\ s^{-1}$), the x and z tensor components of the nitroxide will be averaged. With this assumption it is possible to derive an expression for the average angle between the steroid long axis and the normal of the bilayer plane,

$$\langle\theta_2\rangle = \arccos\left[-\frac{4S_3 + 1}{3}\right]^{\frac{1}{2}} \tag{2}$$

Because of the simple theory used for the analysis of the resonance spectra, the derived values of S_3 and $\langle\theta_2\rangle$ cannot be regarded as absolute numbers, but are extremely useful for comparative purposes.

RESULTS

The spectra of 12-doxylstearic acid and 3-doxylcholestane in fully hydrated egg lecithin multibilayers are shown in Fig. 2 The spectra of 12-doxylstearic acid are

almost angular independent, (S_3 = 0.02), indicating the phospholipid chains in the vicinity of the nitroxide are in a very fluid state. However, when the bilayers were hydrated with an atmosphere of 81 % relative humidity, the spectra of the label were very similar, (S_3 approx. 0.20), to those reported by Jost *et al.*[27] for the same degree of hydration. This indicates there is a significant difference in the fluidity of the chains between the semi- and fully-hydrated states of the bilayers. In contrast to the motion of the stearic acid label, the spectra of 3-doxylcholestane indicate preferential alignment of the steroid nucleus in the hydrated bilayers. The preferential alignment of the steroid spin label is probably due to the fluidity gradient in the bilayers[24–27] and the rigid rod-like shape of the label. The average angle between the steroid long axis and the normal of the bilayers, $\langle\theta_2\rangle$, was calculated to be approximately 31°. It is interesting to note that Seelig[24] found $\langle\theta_2\rangle$ values of 20–25° for 3-doxyl-5α-androstane and 3-doxyl-5α-androstane-17-ol in a model system of sodium decanoate–decanol–water.

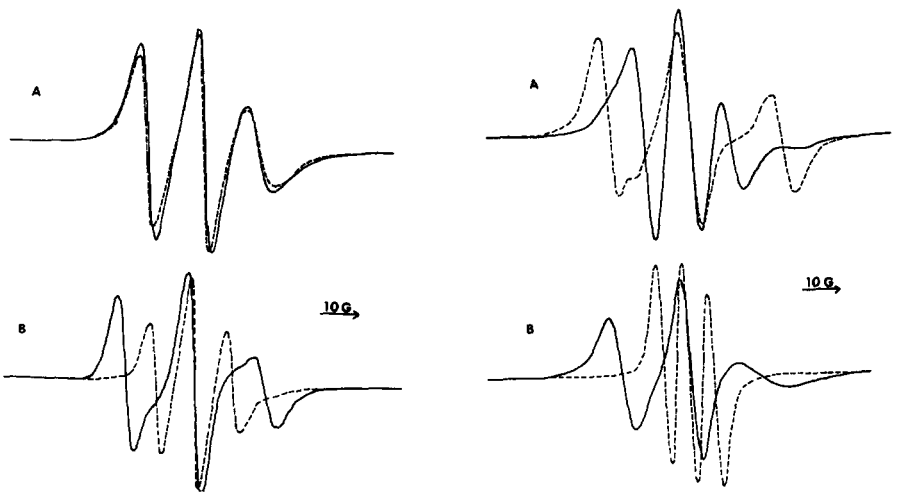

Fig 2 Resonance spectra of (A) 12-doxylstearic acid, and (B) 3-doxylcholestane in hydrated egg lecithin multibilayers The spectra were recorded at room temperature with the magnetic field parallel (-------) and perpendicular (———) to the direction normal to the plane of the multibilayers. The relative positions and heights of the spectra are arbitrary

Fig 3. Resonance spectra of (A) 12-doxylstearic acid, and (B) 3-doxylcholestane in hydrated egg lecithin multibilayers containing 50 mole% cholesterol. The spectra were recorded at room temperature with the magnetic field parallel (--------) and perpendicular (———) to the direction normal to the plane of the multibilayers. The relative positions and heights of the spectra are arbitrary.

The spectra of both labels in hydrated egg lecithin bilayers containing 50 mole % cholesterol are represented in Fig. 3. The most dramatic changes are observed in the 12-doxylstearic acid spectra. From the spectra an order parameter of 0.44 was derived which indicates an increased alignment of the stearic acid long axis parallel to the normal of the bilayers. The deviation angle, $\langle\theta_2\rangle$, of 3-doxylcholestane was calculated to be approximately 8° in the bilayers containing 50 mole % cholesterol. The decrease in the amplitude of motion of both spin labels when cholesterol is added to the multibilayers (reflected by an increase in S_3 for 12-doxylstearic acid and decrease

in $\langle\theta_2\rangle$ for 3-doxylcholestane) is consistent with a decrease in the molecular motion of the phospholipids in the presence of this sterol.

S_3 and $\langle\theta_2\rangle$ as functions of cholesterol content in the phosphatidylcholine multibilayers are shown in Figs 4 and 5. The results indicate that the maximum ordering effect detected by the spin labels occurs at around 33 mole % cholesterol. A value of $S_3 = 0.41$ for 12-doxylstearic acid in the multibilayers containing 33 mole % cholesterol compares favorably with a value of approximately 0.48 in egg lecithin liposomes containing an equivalent amount of cholesterol reported by Hubbell and McConnell[25].

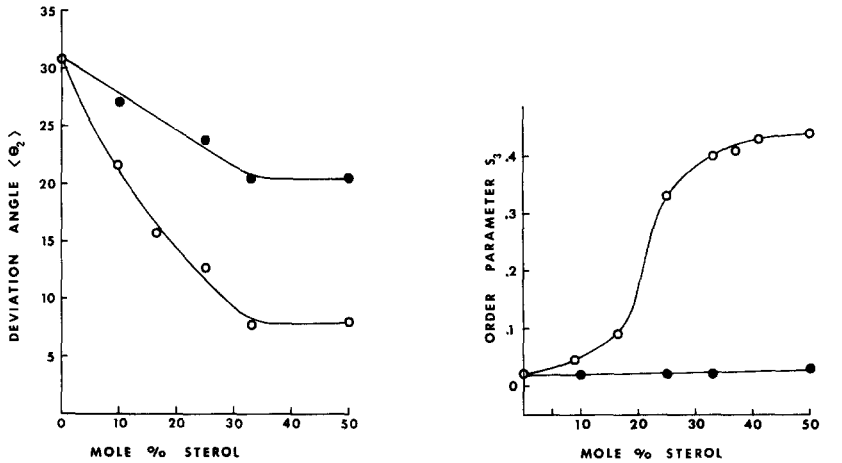

Fig 4 Variation in $\langle\theta_2\rangle$ of 3-doxylcholestane with cholesterol (○—○) and epicholesterol (●—●) content in hydrated egg lecithin multibilayers

Fig 5 Variation in S_3 of 12-doxylstearic acid with cholesterol (○—○) and epicholesterol (●—●) content in hydrated egg lecithin multibilayers

The S_3 and $\langle\theta_2\rangle$ values obtained from hydrated egg lecithin multibilayers containing the cholesterol derivatives are summarized in Tables I and II. All the steroid derivatives affected the motion of 3-doxylcholestane to some degree, while 12-doxylstearic acid was much more sensitive to changes in steroid structure.

Cholesterol, lathosterol and 7-dehydrocholesterol differ from cholesterol in the number and position of double bonds in the B-ring, cholestanol having a saturated B-ring, lathosterol a double bond at C-7–C-8, and 7-dehydrocholesterol two double bonds at C-5–C-6 and C-7–C-8. Both probes indicate that these B-ring derivatives are as effective ordering agents as cholesterol. Demel *et al.*[31] have found that the maximum solubility of 7-dehydrocholesterol in egg lecithin liposomes is 33 mole %. It is not known at present what the solubility limit of this sterol is in oriented multibilayers. We have observed a linear decrease in $\langle\theta_2\rangle$ for 3-doxylcholestane over the range 10–50 mole% of 7-dehydrocholesterol which suggests that 50 mole% can be accommodated in the planar multilayers.

Cholesteryl methyl ether, cholesteryl chloride and 4-cholesten-3-one, which represent C-3 derivatives, have essentially no effect on the motion of 12-doxylstearic acid in the bilayers. Cholesteryl methyl ether or cholesteryl chloride at a concentration of 25 mole% can decrease $\langle\theta_2\rangle$ of 3-doxylcholestane by about 10° which is less

TABLE I

ORDER PARAMETERS OF 12-DOXYLSTEARIC ACID IN HYDRATED PHOSPHATIDYLCHOLINE MULTIBILAYERS

Steroid	*Mole%*	$T'_{\parallel}$	$T'_{\perp}$	T_{zz}	T_{xx}	S_3
Cholesterol	0	14 0	13.5	29 7	5 6	0 02
	25	20.2	11 7	31 6	5 9	0 33
	50	22 0	10 6	31 3	5 6	0 44
Cholesteryl methyl ether	25	14.4	14.0	30 8	5 6	0.01
	50	14 0	13 4	29 6	5 5	0 02
Cholesteryl chloride	50	14 0	13 7	30 0	5 6	0 01
Thiocholesterol	9	14.5	13 3	29 8	5 6	0 04
4-Cholesten-3-one	33–50	15 0	13 2	30 0	5 6	0 07
Epicholesterol	10–50	14.2	13 3	29 6	5.5	0.03
Cholestanol	50	21.7	10 6	31 1	5 8	0 43
Lathosterol	50	22.3	11 6	32 4	6 1	0 42
7-Dehydrocholesterol	50	22.6	10 7	31 9	6 0	0 45
Ergosterol	25–50	14 0	13.1	29 2	5 4	0 03
β-Sitosterol	33–50	15.1	12 9	29 7	5 5	0 09
5α-Androstane-3β-ol	10–50	13 7	13 5	29 5	5.5	0 01
5α-Androstane-3β-ol-17-one	10–50	13 8	13 2	29 2	5 4	0 02

TABLE II

MEAN DEVIATION ANGLE, $<\theta_2>$, OF 3-DOXYLCHOLESTANE IN HYDRATED EGG PHOSPHATIDYLCHOLINE MULTIBILAYERS

Steroid	*Mole%*	$T'_{\parallel}$	$T'_{\perp}$	T_{zz}	T_{xx}	$<\theta_2>$
Cholesterol	0	9 5	17.5	32 3	6 0	30 7
	25	6 8	19 2	32 8	6.1	12 6
	50	6 6	19.9	33 7	6 3	8 1
Cholesteryl methyl ether	25	8 6	17 8	32 1	6 0	26 3
Cholesteryl chloride	50	8 6	17.6	31 8	5 9	26 8
4-Cholesten-3-one	33–50	8.4	18 3	32 7	6.1	26 4
Thiocholesterol	9	—	—	—	—	—
Epicholesterol	25	8.3	18 3	32.6	6 1	23 8
	50	7 8	18 7	32 8	6 1	20 5
Cholestanol	50	6 6	19 5	33 1	6 2	8 5
Lathosterol	50	6 6	20.2	34 0	6 4	6.5
7-Dehydrocholesterol	33	7 5	18 9	32 9	6.1	18.4
	50	6 7	20 2	34 2	6 4	9.1
Ergosterol	25–50	7.4	18 9	32 8	6 1	17.7
β-Sitosterol	33	7.8	18.9	33 1	6 2	20.0
	50	7 4	18 8	32 7	6 1	18 0
5α-Androstane-3β-ol	33	9 1	18 2	33 0	6 2	27 5
	50	7 9	18.8	33 0	6 2	20 7
5α-Androstane-3β-ol-17-one	10	9 3	17 8	32 6	6 1	29 2
	50	10 2	17 3	32 5	6.1	33 7

of an ordering effect than 25 mole % cholesterol. Increasing the concentration of these steroids above 25 mole % in multilayers containing 3-doxylcholestane results in the appearance of spectra of strongly immobilized labels, in addition to the spectra of labels in the lamellar phase. The "strongly immobilized" spectra could only arise from spin labels intercalated in a separate polycrystalline steroid phase. These observations suggest a maximum solubility of 25 mole % of cholesteryl methyl ether

or cholesteryl chloride in the lecithin bilayers. Oldfield and Chapman[13] have determined that these steroids have a very low solubility in lecithin liposomes using phase contrast microscopy. 12-Doxylstearic acid is insensitive to a separate phase of these steroids presumably due to its preferential solubility in the lamellar phase The maximum effect of 4-cholesten-3-one on the motion of 3-doxylcholestane occurs at about 33 mole % steroid and it is much less effective than the same concentration of cholesterol. Demel *et al.*[31] find a solubility limit of 37 mole % for this steroid in liposomes.

Huang *et al.*[32] have demonstrated that thiocholesterol can be incorporated into egg lecithin bilayer vesicles up to a concentration of about 9 mole%. From titration and spin labelling experiments the authors concluded that the thiol groups were in close proximity to the bilayer–water interface Preparation of oriented egg lecithin bilayers containing 9 mole % thiocholesterol and 3-doxylcholestane resulted in a rapid decrease in the paramagnetic resonance signal intensity, which was not detectable after a very short period of time Hydrogen donors such as sulfhydryl groups are known to react with nitroxides forming a diamagnetic hydroxylamine[33]. In contrast, 12-doxylstearic acid was quite stable in films containing 9 mole % thiocholesterol, presumably due to the localization of the nitroxides in the interior of the bilayers The thiocholesterol had the same effect on the motion of the probe as an equivalent amount of cholesterol In films containing the stearic acid label and 50 mole % thiocholesterol, some of the nitroxides were strongly immobilized, indicating the existence of a separate steroid phase

Demel *et al.*[31] report a solubility of 25 mole % epicholesterol in liposomes In the oriented multibilayers we observe an effect on the motion of 3-doxylcholestane up to approximately 33 mole % epicholesterol (Fig 4). The decrease in $\langle\theta_2\rangle$ is much less than that observed with cholesterol. In contrast 10–50 mole % epicholesterol has essentially no effect on the motion of 12-doxylstearic acid (Fig. 5).

Ergosterol and β-sitosterol are plant sterols which represent cholesterol derivatives with structural changes in the hydrocarbon side chain. Ergosterol has two more double bonds than cholesterol (C-7–C-8 and C-22–C-23) and a methyl group at C-24. β-Sitosterol differs from cholesterol by the addition of an ethyl group at C-24. The maximum ordering effect of ergosterol as measured by 3-doxylcholestane is reached at 25 mole % which is in good agreement with its solubility limit in liposomes obtained by Demel *et al.*[31]. The decrease in $\langle\theta_2\rangle$ induced by 25 mole % ergosterol is less than that caused by cholesterol. 25–50 mole % ergosterol has essentially no effect on the motion of 12-doxylstearic acid. Bruckdorfer *et al.*[34] report a solubility limit of 33 mole % β-sitosterol in egg lecithin liposomes. In the oriented multibilayers β-sitosterol causes a linear decrease in $\langle\theta_2\rangle$ of 3-doxylcholestane up to 50 mole % sterol. However, at either 33 or 50 mole % β-sitosterol the degree of order is much less than that induced by cholesterol. At 33–50 mole % β-sitosterol there is a slight influence on the motion of 12-doxylstearic acid, increasing S_3 to approximately 0.1.

5α-Androstane-3β-ol, a derivative which lacks a hydrocarbon side chain, has a slight effect on the motion of 3-doxylcholestane up to 50 mole %, decreasing $\langle\theta_2\rangle$ by about 10°. However, this sterol has no effect on the motion of 12-doxylstearic acid. Another C-17 derivative 5α-androstane-3β-ol-17-one, where the aliphatic side chain is replaced by a polar oxygen atom has no ordering effect on the motion of either spin label.

The effects of the steroids on phospholipid mobility were also investigated using egg lecithin dispersions containing 12-doxylstearic acid The results were qualitatively the same as those obtained in the oriented multibilayers. Only in liposomes containing cholesterol, cholestanol, 7-dehydrocholesterol or lathosterol was the motion of the label sufficiently anisotropic to permit an estimation of the order parameter.

DISCUSSION

Both spin labels provided self-consistent information concerning the steroid structural requirements although 3-doxylcholestane was a less sensitive probe. This can be attributed to differences in the structure and location of each spin label in the bilayers Because the cholestane spin label has a spatial position in the bilayers with the nitroxide close to the membrane–water interface, the main influence on its motion due to added cholesterol arises from the effect of the sterol nucleus on phospholipid chain motion. Therefore, it is not surprising that all the steroid derivatives (except 5α-androstane-3β-ol-17-one) can decrease the random motion of the label to some extent. The oxazolidine ring of the flexible 12-doxylstearic acid label is located in the hydrocarbon interior of the bilayers. Examination of molecular models suggests that the motion of the nitroxide moiety of the fatty acid label should be influenced mainly by the effects of the cholesterol hydrocarbon tail when this sterol is added to the bilayers Thus modification of the C-3 or C-17 substituent which disrupts hydrophobic interactions between the sterol and phospholipid fatty acid chains should have a more pronounced effect on the motion of 12-doxylstearic acid compared to the steroid spin label.

The techniques of monolayer surface pressure measurements[15,16], X-ray diffraction[17,18,35], nuclear magnetic resonance[36,37], ESR spin labelling[10–12] and permeability studies[19] have established that cholesterol inhibits the random motion of phospholipids which are above their gel–liquid crystalline transition temperature. Previous studies by Bruckdorfer *et al.*[38] indicated a relationship between steroid structure and influence on the permeability properties of red blood cell membranes. The results obtained with the 3-doxylcholestane label, (Table II) concur with the preliminary results of Butler *et al* [12] on the effects of various steroids in multibilayers prepared from the lipids of the white matter of bovine brain and the lipids of human erythrocyte ghosts. Our observations are in excellent agreement with the results and conclusions of Demel *et al.*[31,39] who have studied the relationship of steroid structure to steroid–phospholipid interactions in monolayers and the effects of steroid structure on the permeability properties of egg lecithin liposomes These authors concluded that the interactions of sterols with phosphatidylcholine is dependent on (1) a planar steroid nucleus (2) C-3 β-hydroxyl group (3) an intact side chain. The present findings indicate that for maximum ordering effect in egg lecithin bilayers, a steroid with a planar nucleus requires a C-3 β-hydroxyl group and a saturated eight carbon chain at C-17. The presence or position of double bonds in the B-ring appears to have little or no bearing on the extent of interaction The necessity of a hydroxyl group in the β configuration for maximum solubility and ordering suggests that polar interactions between sterol and phospholipid involve stereospecific hydrogen bonding. A hydrogen bond between cholesterol and lecithin in the dry state has been detected by infrared spectroscopy[40]. The specific structural requirements of the C-17 substituent for maximum ordering

effect indicate definite hydrophobic interactions between the hydrocarbon tail of the sterol and the phospholipid fatty acid chains. The primary functions of the steroid nucleus seem to be: (1) to maintain the correct separation between the hydroxyl group and the hydrocarbon side chain, for maximum polar and hydrophobic interactions with the phospholipids (2) to provide rigidity to the sterol:phospholipid "complex". Hydrophobic interactions involving the steroid nucleus may also be important. It should be noted that steroid–phospholipid interactions are also dependent on the chemical structure of the phospholipid. We have found that the structural requirements of a membrane ordering steroid differ in egg lecithin and bovine brain sphingomyelin bilayers (R. A. Long, unpublished results).

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REFERENCES

1 Griffith, O. H. and Waggoner, A. S (1969) *Acc Chem Res* 2, 17
2 McConnell, H. M. and McFarland, B G (1970) *Quart Rev Biophys.* 3, 91
3 Smith, I C P (1971) in *Biological Applications of Electron Spin Resonance Spectroscopy* (Bolton, J. R , Borg, D and Swartz, H , eds) Wiley–Interscience, New York
4 Jost, P , Waggoner, A. S and Griffith, O H (1971) in *Structure and Function of Biological Membranes* (L I Rothfield, ed) Academic Press, New York
5 Mehlhorn, R J and Keith, A D (1971) in *Membrane Molecular Biology* (Fox, C F. and Keith, A D , eds) Sinauer Associates, Stamford, Connecticut
6 Hubbell, W L and McConnell, H. M (1968) *Proc. Natl Acad. Sci U S.* 61, 12
7 Barratt, M D , Green, D K and Chapman, D (1969) *Chem Phys Lipids* 3, 140
8 Waggoner, A S , Kingsett, T. J , Rottschaefer, S , Griffith, O H and Keith, A D. (1969) *Chem Phys Lipids* 3, 245
9 Hsia, J C , Schneider, H and Smith, I C P. (1970) *Chem Phys Lipids* 4, 238
10 Hsia, J C , Schneider, H and Smith, I C P. (1970) *Biochim Biophys Acta* 202, 399
11 Long, R A , Hruska, F E , Gesser, H D , Hsia, J C and Williams, R. (1970) *Biochem Biophys. Res. Commun* 41, 321
12 Butler, K W , Smith, I C P. and Schneider, H (1970) *Biochim Biophys Acta* 219, 514
13 Oldfield, E and Chapman, D (1971) *Biochem. Biophys Res. Commun.* 43, 610
14 Hsia, J C , Schneider, H and Smith, I C P (1971) *Can J Biochem* 49, 614
15 Bernard, L de (1958) *Bull Soc Chim Biol* 40, 161
16 Demel, R A , van Deenen, L L M and Pethica, B A. (1967) *Biochim Biophys Acta* 135, 11
17 Lecuyer, H and Dervichian, D G (1969) *J. Mol Biol* 45, 39
18 Levine, Y. K. and Wilkins, M H F (1971) *Nature, New Biol* 230, 69
19 Gier, J. de, Mandersloot, J G and van Deenen, L L M (1968) *Biochim Biophys Acta* 150, 666
20 McElhaney, R N , Gier, J de and van Deenen, L L M (1970) *Biochim Biophys Acta* 219, 245
21 Keana, J F W , Keana, S B and Beetham, D (1967) *J Am Chem Soc.* 89, 3055
22 Hubbell, W L and McConnell, H. M (1969) *Proc Natl Acad Sci U S* 63, 16
23 Libertini, L J , Waggoner, A S , Jost, P C and Griffith, O H (1969) *Proc. Natl Acad. Sci. U S* 64, 13
24 Seelig, J (1970) *J Am Chem Soc* 92, 3881
25 Hubbell, W L. and McConnell, H M (1971) *J Am. Chem Soc* 93, 314
26 McFarland, B G and McConnell, H M (1971) *Proc Natl Acad Sci. U S.* 68, 1274
27 Jost, P , Libertini, L J , Hebert, V G and Griffith, O H (1971) *J Mol Biol* 59, 77
28 McConnell, H M (1967) in *Magnetic Resonance in Biological Systems* (Ehrenberg, A , Malmstrom, B G and Vanngård, T , eds) Pergamon Press, Oxford
29 Saupe, A , Englert, G and Pova, A (1967) *Adv Chem* Ser No 63
30 Ferruti, P , Gill, D , Harpold, M A and Klein, M P (1969) *J Chem Phys.* 50, 4545
31 Demel, R A , Bruckdorfer, K R and van Deenen, L L M (1972) *Biochim Biophys Acta* 255, 321
32 Huang, C , Charlton, J P , Shyr, C I and Thompson, T E (1970) *Biochemistry* 9, 3422

33 Rozantsev, E G (1970) *Free Nitroxyl Radicals*, Plenum Press, New York
34 Bruckdorfer, K R , Graham, J M and Green, C. (1968) *Eur. J. Biochem.* 4, 512
35 Rand, R. P and Luzzati, V (1968) *Biophys. J* 8, 125
36 Chapman, D and Penkett, S. A (1966) *Nature* 211, 1304
37 Darke, A., Finer, E. G , Flook, A. G and Phillips, M C (1971) *FEBS Lett.* 18, 326
38 Bruckdorfer, K R., Demel, R A , de Gier, J. and van Deenen, L L. M. (1969) *Biochim. Biophys Acta* 183, 334
39 Demel, R A , Bruckdorfer, K. R. and van Deenen, L L. M (1972) *Biochim. Biophys. Acta* 255, 311
40 Zull, J. E., Greanoff, S. and Adam, H K (1968) *Biochemistry* 7, 4172